

Human Immunodeficiency Virus Type 1 Clones Chimeric for the Envelope V3 Domain Differ in Syncytium Formation and Replication Capacity

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Chimeric human immunodeficiency virus type 1 (HIV-1) molecular clones differing only in the envelope V3 region were constructed. The V3 regions were derived from two HIV-1 isolates with a non-syncytium-inducing, non-T-cell-tropic phenotype and from four HIV-1 isolates with a syncytium-inducing, T-cell-tropic phenotype. When assayed in SupT1 cells, the two chimeric viruses with a V3 region derived from the non-syncytium-inducing isolates did not induce syncytia and showed a low level of replication. The four chimeric viruses with a V3 region derived from the syncytium-inducing isolates did induce syncytia and replicated efficiently in SupT1 cells. In A3.01 cells, which do not support syncytium formation, the V3 loop affected replication similarly. Upon prolonged culture in SupT1 cells, the phenotype of a non-syncytium-inducing, low-replicating chimeric HIV-1 converted into a syncytium-inducing, high-replicating phenotype. Mutations within the usually conserved GPGR tip of the loop, which were shown to be responsible for the conversion into the syncytium-inducing, high-replicating phenotype, had occurred. In vitro mutagenesis showed that coupled changes of amino acids at both sides of the tip of the V3 loop were able to convert the viral phenotype from non-syncytium-inducing, low replicating into syncytium inducing, high replicating. Our data show that the V3 loop is involved in both syncytium forming and replicative capacity of HIV-1.

The third variable region (V3) of the external glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) contains a binding site for human and experimentally induced antibodies that are able to neutralize HIV-1 infection as well as block syncytium formation in vitro (9, 15, 23, 26, 27). The V3 domain is located between amino acids 296 and 331 of gp120 of HIV-1 and has a type 2 β -turn conserved secondary structure, which is induced by the amino acids GPGR. Mapping of the disulfide bonds of gp120 has revealed that the cysteine residues bordering the V3 region are linked, giving the V3 region a loop structure (20).

The gp120 V3 loop is capable of eliciting isolate-specific neutralizing antibodies in both HIV-1-infected humans and animals (9, 12-15, 21, 23, 26, 27). Zwart et al. (36), Devash et al. (6), and LaRosa et al. (18) have shown the immunodominance of the V3 region. Moreover, the V3 loop contains an epitope recognized by cytotoxic T lymphocytes of mice (32) and humans (4). Besides serving as a major target for the immune system, the V3 loop probably has additional functions in viral replication. Evidence that the V3 loop has a function in viral replication has been presented by Takeuchi et al. (33), who showed that a single amino acid substitution in the tip of the V3 loop is responsible for the altered host range of an HIV-1 isolate. In addition, O'Brien et al. (25) have shown that the HIV-1 tropism for mononuclear phagocytes can be determined by a region of gp120 which includes the V3 loop. In vitro mutagenesis of the V3 loop has indicated that it is involved in the HIV-1 envelope-mediated fusion of CD4-positive HeLa cells (8).

In order to facilitate a detailed study of the function of the V3 loop in HIV-1 infection, we have developed a cloning

system which enables us to exchange and mutagenize the V3 loop in an HXB-2 background. As a starting point, we used V3 regions derived from six different HIV-1 isolates: three viruses of which two are non-syncytium-inducing in primary T cells, isolated at different time points from a single individual, and the three HIV-1 viruses MN, RF, and SF2. The phenotypic differences of the resultant recombinant viruses varying only in their V3 loop indicates that the V3 loop affects the capacity to form syncytia in SupT1 cells and the level of replication in SupT1 and A3.01 cells.

MATERIALS AND METHODS

Source of V3 regions. Individual 168 of the Amsterdam cohort (7), showing the emergence of HIV core antigen in serum and progressing to AIDS, was selected for the present study (Table 1) (168 was designated number 2 by Tersmette et al. [34]). Sequential HIV-1 isolates were obtained by cocultivation with peripheral blood lymphocytes (PBLs). The replication rate of the isolate as well as the capacity to induce syncytia was determined. Sequential virus isolates showed a switch from the non-syncytium-inducing to the syncytium-inducing phenotype during the progression to disease. The V3 regions 168.1 and 168.3 were derived from non-syncytium-inducing, non-T-cell-tropic virus isolates. The V3 region 168.10 was derived from a syncytium-inducing, T-cell-tropic virus isolate. V3 regions were also derived from H9 cells persistently infected with HIV-1 isolate MN, RF, or SF2.

Direct sequencing. DNA from PBLs infected with the viral isolates or from infected H9 cells was isolated according to the method of Boom et al. (1). Samples (100 ng) of DNA were amplified by the polymerase chain reaction (PCR). Primers used to amplify the V3 region were J-5'-2-ksi (5'-

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TABLE 1. Sequential clinical and virological data on patient 168

Isolate	Follow-up (mo)	Clinical classification (CDC stage)	Immunological status (CD4 ⁺ cells [10 ⁹ /liter])	Serological status		Virus characteristics				
				Core antigen (pg/ml)	Antibody (titer)	Syncytia in PBLs	Host range			Replication rate
							PBL	H9	U937	
168-1	0	II/III	0.2	NT	2	—	+	—	—	++
168-2	3	II/III	NT ^a	49	1	—	+	—	—	++
168-3	6	II/III	0.3	82	1	—	+	—	—	++
168-4	9	II/III	0.6	117	1	—	+	—	—	++
168-5	12	II/III	0.4	99	1	+	+	+	+	+++
168-7	18	II/III	0.3	185	1	+	+	+	+	+++
168-10	27	IV C-1	0.0	NT	1	+	+	+	+	+++

^a NT, not tested.

ATAAGCTTGCAGTCTAGCAGAAGAAGA-3'; HXB-2 positions 6558 to 6576 [24]), containing an additional *Hind*III site, and J-3'-2-ksi-2 (5'-ATGAATTCTGGGTCCCCTCCTG AGGA-3'; positions 6860 to 6880), containing an additional *Eco*RI site. The PCR mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.8 mM MgCl₂, 0.01% gelatin, 0.2 mM (each) deoxynucleoside triphosphates, 10 pmol of each oligonucleotide primer, and 2 U of *Taq* polymerase (a gift from Perkin-Elmer Cetus) in a final volume of 100 µl. The reaction was performed for 35 cycles. Each cycle consisted of a 1-min denaturation step at 95°C, a 1-min annealing step at 55°C, and a 2-min elongation step at 72°C. After 35 cycles, the reactions were extended for another 10 min at 72°C. The amplified fragment was purified by preparative gel electrophoresis. The amplified DNA was made single stranded by performing an additional PCR for 15 cycles in the presence of only one primer. The single-stranded DNA was sequenced using the dideoxy chain termination method and the complementary primer. Parallel to the directly obtained sequence, a second PCR, using the same primers, was performed on the original DNA. These PCR products were digested with *Eco*RI and *Hind*III and cloned in pGEM7 (Promega Biotec). Clones were sequenced and used to confirm the direct sequences.

Molecular clones. For the construction of molecular clones with different V3 regions, two plasmids were generated.

(i) **pJJ5.** Plasmid pJJ5 is HXB-2 (with flanking cellular sequences) (30) from which the *Nco*I-to-*Bam*HI fragment (nucleotides 5221 to 8026) had been replaced by a stuffer fragment cloned in pSP73 (Promega).

(ii) **pJJ25.** Plasmid pJJ25 contains the *Nco*I-to-*Bam*HI fragment of HXB-2 (positions 5221 to 8026) cloned into a pSP73 derivative in which an *Nco*I site has been inserted between the *Kpn*I site and the *Sma*I site of the polylinker. To facilitate cloning of various V3 regions into the HXB-2 *Nco*I-to-*Bam*HI fragment, sequences from just upstream (*Pvu*II site, position 6629) to just downstream (position 6770) from the V3 region were deleted and replaced by the *Pvu*II-to-*Xba*I part of the pSP73 polylinker. Introduction of the *Xba*I site downstream of the V3 region was performed by PCR and conserves the encoded amino acid sequence.

Cloning strategy. PCR was performed on 100 ng of DNA of a pGEM7 clone containing a patient V3 region (which was used for the confirmation of the direct sequences) and on DNA isolated from H9 cells chronically infected with MN, RF, and SF2. Primers used to amplify the V3 domain were 5'*Pvu*II-J (5'-GTACAGCTGAATGAATCTGTAGAAATTAA TTGT-3'; positions 6629 to 6658) and 3'*V3-Xba*I (5'-CCATT NTG[T/C]TCTAGAAAGGTTACA-3'; positions 6761 to 6784). Both primers end with the TGT/ACA triplet encoding the

cysteines bordering the V3 region, thereby amplifying just the V3 region and preventing differences outside the V3 region from being incorporated in the molecular clone. PCR conditions were as described above, except that 2.0 mM MgCl₂ was used. The PCR products were purified by gel electrophoresis, digested with *Pvu*II and *Xba*I, and cloned into *Pvu*II- and *Xba*I-digested pJJ25. The V3 chimeric *Nco*I-*Bam*HI fragments of pJJ25 were obtained by digestion and cloned into pJJ5, creating the molecular HXB-2 clones with different V3 regions (Fig. 1).

Mutagenesis of V3 constructs. Mutations which replace the 168.1 sequence by the MN sequence were introduced in the 168.1 V3 region. In order to avoid contamination by nonmutated pJJ168.1-25 sequences in the mutagenesis procedure, plasmid pJJ168.1-25 (pJJ25 containing the V3 region of 168.1) was digested in two separate digestions with *Rsa*I (position 6656) and with *Mae*III (position 6762). Fragments smaller than 560 bp were excised from an agarose gel and purified. These DNAs served as templates for constructing mutant 168.1 V3 regions by PCR. The procedure to create the mutant R-168.1 containing an R instead of an S at amino acid position 306 was as follows (Fig. 2). The *Rsa*I-digested pJJ168.1-25 was amplified by PCR using the 5'-168.1-R primer (5'-GAAAAAGGATACATATAGG-3'; positions 6681 to 6705) and 3'*V3-Xba*I primer to obtain the R-168.1-*Xba*I fragment. The *Mae*III-digested pJJ168.1-25 was amplified by PCR using the 5'*Pvu*II-J primer and 3'-168.1-R primer

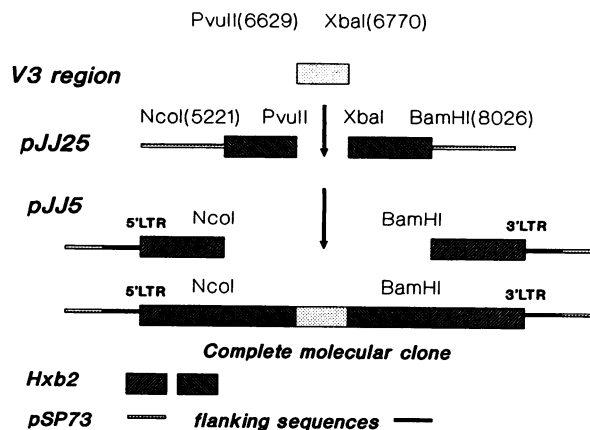


FIG. 1. Construction of molecular clones with different V3 regions. The V3 region (open bar) was obtained by PCR and cloned into a V3-deleted *Nco*I-*Bam*HI fragment of HXB-2. Subsequently, the *Nco*I-*Bam*HI fragment was used to reconstitute an infectious molecular HXB-2 clone (see Materials and Methods).

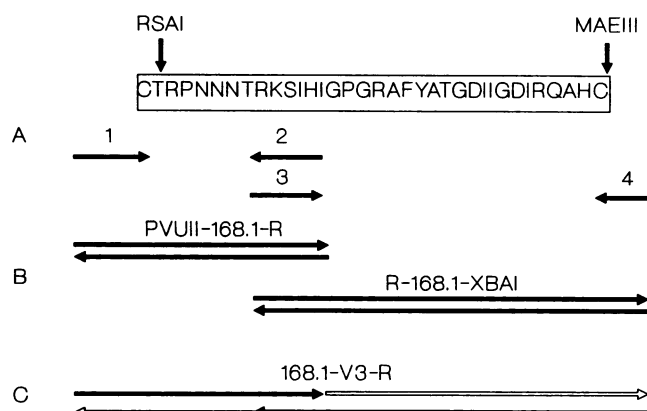


FIG. 2. Construction of the R mutant (position 306) of the 168.1 V3 region. (A) Location and direction of the primers used as well as the positions of the restriction enzymes *MaeIII* and *RsaI*. Primers: 1, 5'PvuII-J; 2, 3'-168.1-R; 3, 5'-168.1-R; 4, 3'V3-XbaI. (B) PCR to obtain the PvuII-168.1-R and R-168.1-XbaI fragments. (C) PCR to obtain the 168.1 V3 region with the R at amino acid position 306 by using the PvuII-168.1-R and R-168.1-XbaI fragments as templates (see Materials and Methods).

(5'-CCTATATGTATCCTTTTTC-3'; positions 6681 to 6705) to obtain the PvuII-168.1-R fragment.

PCR conditions were as described above, except that 4.0 mM MgCl₂ was used. All the fragments were gel purified. To obtain a 168.1 V3 region with the R at position 306, a PCR with the 5'PvuII-J and the 3'V3-XbaI primers was performed using PCR conditions as described above and 5 ng of the PvuII-168.1-R and R-168.1-XbaI fragments as templates. The PCR-derived R-168.1 V3 region was digested with PvuII and XbaI and cloned into PvuII-XbaI-digested pJJ25, creating pR-168.1-25. Subsequently, a viral molecular clone containing the R-168.1 V3 region was created by excision of the *NcoI*-to-*BamHI* fragment from pR-168.1-25 and cloning pR-168.1-25 into *NcoI*-to-*BamHI*-digested pJJ5.

A similar protocol was used to obtain a 168.1-V3 region encoding the amino acid stretch TTKN instead of ATGD at amino acid positions 317 to 320. Primers used were 5'-168.1-TTKN primer (5'-TATACAACAAAAATATAATAGG-3'; positions 6722 to 6741) and 3'-168.1-TTKN primer (5'-CCTATTATTTTTTGTGTATATAAATG-3'; positions 6717 to 6741). For the construction of the mutant containing both the R

and TTKN mutations, the pJJ168.1-25 derivative encoding the TTKN stretch served as a template and a procedure identical to that for creating pR-168.1-25 was used.

Transfections. Five micrograms of cesium chloride gradient-purified DNA of a viral molecular clone was electroporated into 5×10^6 SupT1 cells and 5×10^6 A3.01 cells. Immediately after electroporation, an additional 5×10^5 SupT1 or A3.01 cells were added. Electroporation conditions, identical for both cell lines, were set at 960 μ F and 250 V (on a Bio-Rad Gene-Pulser).

SupT1 cells were kindly provided by J. Hoxie, and A3.01 cells were obtained from T. Folks via the NIH AIDS Research and Reference Reagent Program. The transfections were maintained in 5 ml of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum and incubated at 37°C in the presence of 5% CO₂. Three days after transfection, an additional 5 ml of medium was added. The replication of the viruses was monitored by the assay of p24 core antigen production (Abbott) and observation of syncytium formation (examined by two observers working independently). The p24 core antigen production was standardized with the positive control from Abbott with a p24 concentration of 200 pg/ml.

RESULTS

Sequence analysis of V3 regions of syncytium-inducing and non-syncytium-inducing viruses. At 3-month intervals, virus was isolated from PBLs of patient 168 after coculturing with donor PBLs. At the start of the study period, patient 168 was asymptomatic (Centers for Disease Control [CDC] stage II/III). At the time point that isolate 168.10 was obtained, AIDS was diagnosed (CDC stage IV C-1).

Direct sequences of the V3 regions of longitudinal viral isolates of patient 168 were obtained and are depicted in Fig. 3. The direct sequences showed minimal differences with individual clones (Fig. 3 and 4a). Five nonsilent mutations in the V3 region occurred during the period of study which provided the V3 region with a higher positive charge (Fig. 4a). We chose the 168.1, 168.3, and 168.10 sequences, as representative of the V3 sequences occurring (Fig. 4a).

The viral isolates 168.1 and 168.3 have the non-syncytium-inducing phenotype, whereas viral isolate 168.10 has the syncytium-inducing phenotype (Table 1). This indicates that the V3 regions of 168.1 and 168.3 are derived from non-

ISOLATE	AMINO ACID SEQUENCE
168.1	CTRPNNNTRKSIHIGPGRAFATGDIIGDIRQAHC
168.2	-----
168.3	-----P-----
168.4	-----P-----
168.5	-----R-----T--Q--N-----
168.7	-----R-----T--Q--N-----
168.10	-----R-----T--Q--N-----

FIG. 3. Deduced amino acids of the direct sequences of the V3 region of sequential isolates from patient 168. Viral isolates were obtained from sequential samples of PBLs of patient 168 by cocultivation with donor PBLs. A V3-specific PCR was performed on DNA isolated from the cocultured PBLs. The dominant nucleotide sequence of the PCR products was obtained by direct sequencing using the dideoxy chain termination method.

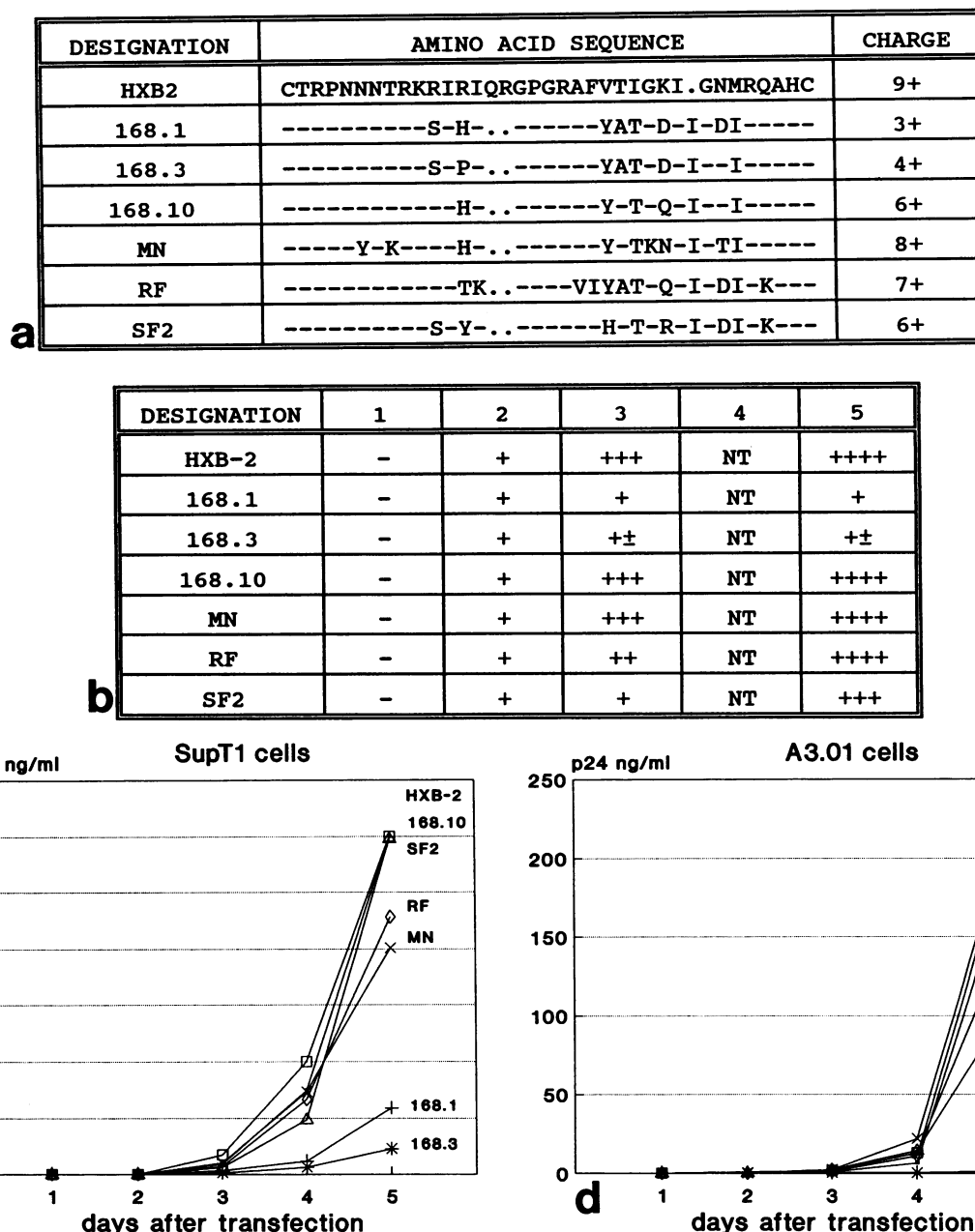


FIG. 4. (a) Amino acid sequence comparison and overall positive charge of the V3 regions of the molecular clones used in this transfection experiment; (b) Relative number of syncytia produced in SupT1 cells during 5 day cultivation of the transfections shown in panel c (1 to 5, days after transfection; NT, not tested); (c and d) p24 core antigen production measured during a 5-day culture period of single transfections using 5×10^6 SupT1 cells (c) or 5×10^6 A3.01 cells (d) and 5 μ g of DNA of the molecular clones bearing the V3 regions shown in panel a.

syncytium-inducing viruses and the V3 region of 168.10 is derived from syncytium-inducing virus.

Moreover, nucleic acid sequences coding for V3 regions were PCR amplified from H9 cells persistently infected with MN, RF, and SF2. Sequence analysis of MN, RF, and SF2 cloned V3 regions showed identity with the published sequences (10, 13, 31), except that for RF a change of an S to an R at amino acid position 308 was observed.

To determine the effect of the observed changes in the V3 region on the phenotype of the virus, the V3 regions were cloned into the HXB-2 molecular clone cassette system,

thereby replacing the HXB-2 V3 region (Fig. 1). After transfection in SupT1 or A3.01 cells, HXB-2 viruses containing the different V3 regions were obtained.

Effect of V3 regions on syncytium formation and replication in SupT1 cells. To check whether our cloning system would give rise to infectious viruses, we first inserted the V3 region of HXB-2 obtained by PCR with the 5'PvuII-J and the 3'V3-XbaI primers into our molecular HXB-2 clone cassette system. By transfection in SupT1 cells, the newly made molecular HXB-2 clone was compared with the original infectious molecular HXB-2 clone (used for constructing our

cloning system). Both molecular clones gave rise to infectious virus with the syncytium-inducing phenotype after transfection to SupT1 cells (data not shown). The molecular HXB-2 clone bearing the V3 region of HXB-2 served as a positive control in all the transfection experiments shown.

Molecular clones containing the V3 regions of 168.1, 168.3, 168.10, MN, RF, SF2, and HXB-2 were transfected in SupT1 cells in three separate experiments. During a 5-day culture period, the p24 antigen level in the culture medium was measured and the capability to form syncytia was determined. Data from one representative experiment are shown (Fig. 4). The different molecular clones behaved similarly until 3 days after transfection. The initially formed syncytia of the chimeric viruses 168.1 and 168.3 were small and did not increase in number or size during the 5-day culture period. These initially formed syncytia of the chimeric viruses 168.1 and 168.3 are probably derived from transfected cells, whereas the quality and/or quantity of the viruses produced is not sufficient to induce additional syncytium formation. In contrast, the chimeric viruses containing the V3 regions of 168.10, MN, RF, SF2, and HXB-2 were able to induce syncytia which increased in size and number during the 5-day culture period. In SupT1 cells, the levels of p24 antigen production of the chimeric viruses 168.1 and 168.3 were low compared with the levels of p24 production of the chimeric viruses 168.10, MN, RF, SF2, and HXB-2. Apparently, the non-syncytium-inducing, low-replicating or syncytium-inducing, high-replicating phenotype of the chimeric virus accords with the phenotype of the viral isolate from which the V3 region is derived. Even the replacement of the HXB-2 V3 region by a V3 derived from an envelope protein differing as much as that of MN conserves the syncytium-inducing, high-replicating phenotype. In A3.01 cells, which we did not observe to support syncytium formation, the levels of p24 antigen production of the chimeric viruses showed the same trend as in SupT1 cells (Fig. 4), with the chimeric viruses 168.1 and 168.3 producing less p24 antigen and more slowly compared with the chimeric viruses 168.10, MN, RF, SF2, and HXB-2.

Analysis of the viruses produced. To verify that all transfections had produced infectious virus, culture medium was filtered and used to infect SupT1 cells. The cell-free transfer of virus showed that in all transfections infectious virus retaining the original phenotype was produced, monitored by measuring p24 antigen production and syncytium formation. To test the stability of the chimeric viruses 5 days after transfection, DNA was isolated from SupT1 cells and the V3 region was cloned and sequenced. Sequence analysis revealed that after short-term cultivation the V3 region was identical to the V3 region of the input molecular clone in all cases.

Phenotypic switch after prolonged cultivation. In two separate transfections in SupT1 cells, when the molecular clones containing the V3 regions of 168.1 and 168.3 were used, the initially observed syncytia disappeared at the end of a 5-day cultivation period. Prolonged cultivation of these transfections showed a reappearance of syncytia at days 10 and 14, respectively. After the reappearance of syncytia, DNA from the transfected cells was isolated. The V3 regions were amplified by PCR and cloned. Sequence analysis of the clones obtained revealed that alterations in the V3 regions had occurred (Fig. 5). The alterations all took place in the highly conserved GPGR tip of the V3 loop. To determine whether the alterations in the V3 regions could be responsible for the observed phenotypic switch, chimeric viral molecular clones containing these V3 regions were gener-

ated and transfected to SupT1 and A3.01 cells (Fig. 5). The molecular clones containing the V3 region of 168.1 with the GQRR and GQRK sequences in the tip of the V3 loop showed a much higher p24 expression level in SupT1 cells. They also formed a larger number of syncytia in SupT1 cells compared with the viral molecular clones containing the V3 region of 168.1 with the GPGR or GPRR sequence and of 168.3 with the GRGR sequence in the tip of the loop. When assayed in A3.01 cells, the tip of the loop mutants containing the GQRR and GQRK sequence only moderately changed the expression level of p24 of the chimeric virus 168.1. This result indicates that point mutations in the highly conserved tip of the loop are viable and can, although dependent on the cell line, alter phenotype of a virus.

Mutagenesis of the V3 region of 168.1. To learn more about the contribution of individual amino acids to the level of replication, we exchanged a few selected amino acids from the V3 region of 168.1 for those of MN (position 306, S → R; positions 317 to 320, ATGD → TTKN; Fig. 2 and 6). The molecular clones bearing the V3 regions of 168.1, R-168.1, TTKN-168.1, R-TTKN-168.1, and MN were transfected to SupT1 and A3.01 cells (Fig. 6). The introduction of the TTKN stretch did not affect the phenotype of the chimeric virus 168.1. The R change in SupT1 cells resulted in a phenotype which was intermediate between those of the chimeric viruses 168.1 and MN in both syncytium induction and replication. In A3.01 cells this observed phenotypic change was less pronounced. The R-TTKN mutations changed the non-syncytium-inducing, low-replicating phenotype of the chimeric virus 168.1 into an syncytium-inducing, high-replicating phenotype (comparable to that of MN). The data derived from the mutagenesis of the V3 region of 168.1 indicate that both sides of the GPGR tip of the loop are involved in determining the phenotype of a chimeric virus.

DISCUSSION

The third variable region (V3) of the envelope glycoprotein gp120 is known to encompass the major neutralizing domain of HIV-1. Disulfide bridge formation of the cysteine residues at the 5' and 3' ends of the V3 region at amino acid positions 296 and 331 on gp120 give this region a loop structure. Binding of antibodies to this loop can give rise to isolate-specific virus neutralization and cell fusion inhibition but does not directly block the binding of the virus to its CD4 receptor. All of these studies (9, 12–15, 20, 21, 23, 26, 27) indicate that the V3 region, despite its high variability, plays an important role in the life cycle of HIV-1. To study the role of the V3 region in HIV-1 infection and to gain insight into the importance of the nucleotide variation observed *in vivo*, we designed a plasmid vector system allowing us to construct HXB-2 viruses which differed only in their V3 region.

The phenotype of the chimeric HXB-2 viruses clearly showed the involvement of the V3 region in replication as monitored by p24 antigen expression. In SupT1 cells and A3.01 cells, chimeric HXB-2 viruses with a V3 region derived from the non-syncytium-inducing isolates 168.1 and 168.3 did not replicate efficiently. Chimeric HXB-2 viruses containing the V3 domain of four syncytium-inducing viruses induced syncytia and had levels of replication comparable to HXB-2. The 168.10, MN, RF, and SF2 viruses as well as their V3 domains are highly divergent from HXB-2 (24). Despite the divergence, the V3 domain of HXB-2 can be substituted by the V3 domains of 168.10, MN, RF, and SF2 without changing the viral phenotype. Apparently, the V3 region can act as an independent protein domain in which

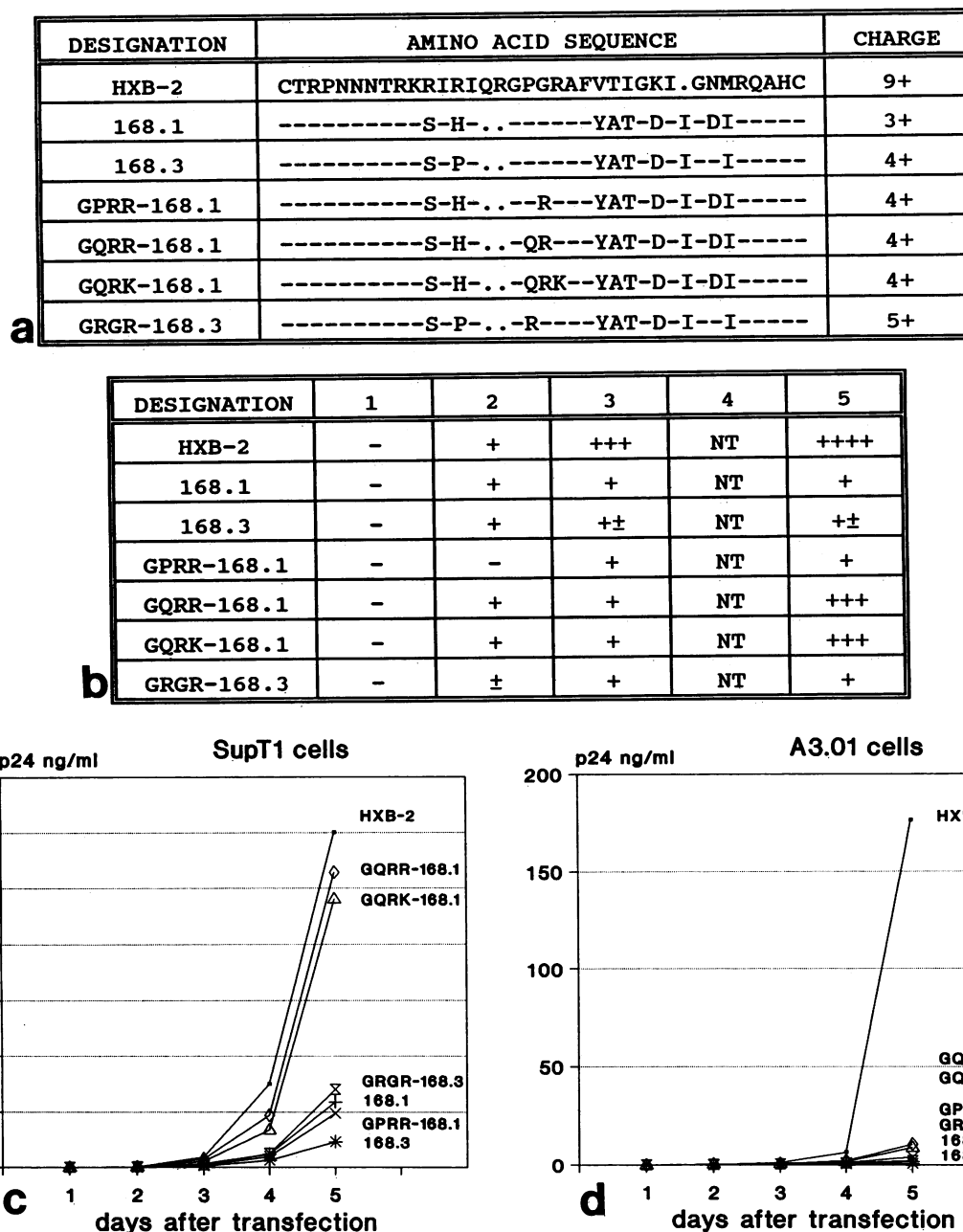


FIG. 5. (a) Amino acid sequence comparison and overall positive charge of the V3 regions of the molecular clones used in this transfection experiment; (b) relative number of syncytia produced in SupT1 cells during 5-day cultivation of the transfections shown in panel c (1 to 5, days after transfection; NT, not tested); (c and d) p24 core antigen production measured during a 5-day culture period of single transfections using 5×10^6 SupT1 cells (c) or 5×10^6 A3.01 cells (d) and 5 μ g of DNA of the molecular clones bearing the V3 regions shown in panel a.

considerable variation is tolerated without abolition of the envelope's functional properties. To some extent the HXB-2 background will also influence the phenotype of the chimeric viruses, as is shown by the chimeric viruses 168.1 and 168.3 containing V3 regions from non-syncytium-inducing viral isolates which cannot normally be propagated in T-cell lines. However, the V3 domains of 168.1 and 168.3 still give rise to chimeric viruses with the non-syncytium-inducing, low-replicating phenotype. These results suggest that not only the V3 region but also other regions of gp120, like the second

conserved region (5, 11, 16, 35), are involved in determining the syncytium-inducing and replicative capacities of HIV-1.

Upon prolonged propagation in SupT1 cells, the non-syncytium-inducing chimeric viruses with the V3 region of 168.1 or 168.3 tended to give rise to virus mutants with a syncytium-inducing and a high replicative capacity. The mutations in the V3 region, which converted the non-syncytium-inducing, low-replicating phenotype of the chimeric virus 168.1 into a syncytium-inducing, high-replicating phenotype, are located in the largely conserved tip of the

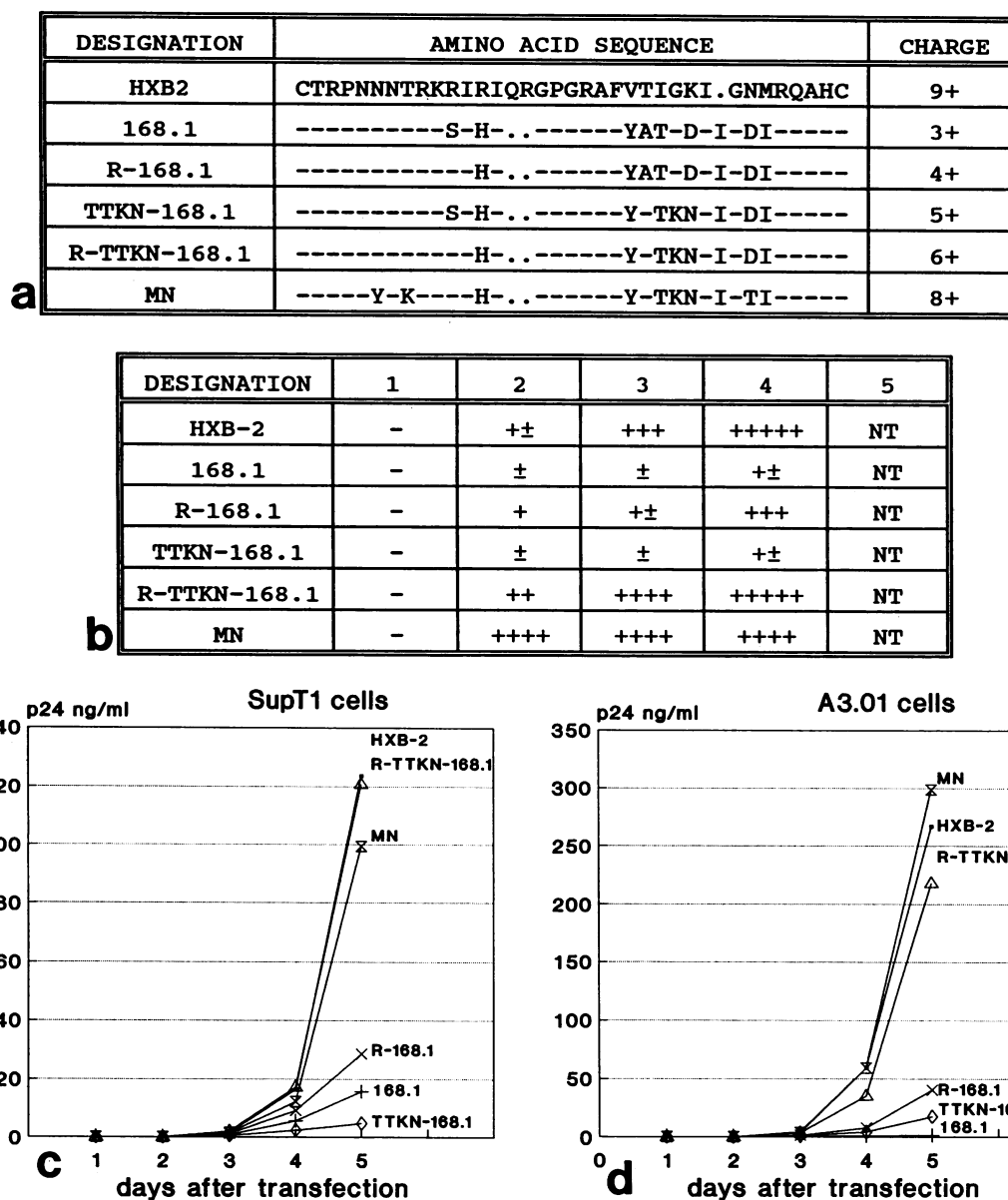


FIG. 6. (a) Amino acid sequence comparison and overall positive charge of the V3 regions of the molecular clones used in this transfection experiment; (b) relative number of syncytia produced in SupT1 cells during 5-day cultivation of the transfections shown in panel c (1 to 5, days after transfection; NT, not tested); (c and d) p24 core antigen production measured during a 5-day culture period of single transfections using 5×10^6 SupT1 cells (c) or 5×10^6 A3.01 cells (d) and 5 μ g of DNA of the molecular clones bearing the V3 regions shown in panel a.

loop (GPGR) (18). In vitro mutagenesis studies by Freed et al. (8) suggested that changes in the tip of the loop of HXB-2 abolish syncytium-inducing capacity. However, our data show that specific changes (GQRR and GQRK; note that the QR combination, albeit at a different position, is present in HXB-2 V3 as well) in the tip of the loop can enhance syncytium-inducing capacity and yield viable virus with accelerated replication. Furthermore, Takeuchi et al. (33) showed that HIV-1 with a GSGR tip of the loop is viable and has an altered host range compared with an identical HIV-1 with a GPGR tip of the loop. In addition, the difference in phenotype between the 168.1 (GPGR) and the GQRR and GQRK chimeric viruses is far less pronounced in A3.01 cells than in SupT1 cells, indicating that the effects of changes in

the V3 region are cell line dependent as well. Masuda et al. (22) have shown that HIV-1 with a GQGR tip of the loop can arise as an escape mutant under pressure of the neutralizing monoclonal antibody 0.5 β . Langedijk et al. (17) showed that the contribution of individual amino acids within the tip of the loop can be essential to the binding of antibodies. In contrast to 168.10, which shows an antigenicity similar to that of 168.1 (36), the tip of the loop mutants are expected to have an altered antigenicity. In conclusion, although largely conserved in vivo, the tip of the V3 loop shows variation which may crucially influence the phenotype and host range of HIV-1.

The V3 sequences flanking the tip of the loop are involved in determining the phenotype as well. Residues of the 168.1

non-syncytium-inducing sequence were replaced by those of the MN sequence on both the amino-terminal and carboxy-terminal sides of the tip of the loop. Although a 1-amino-acid substitution (position 306, S to R) at the amino-terminal side of the loop already had an enhancing effect on syncytium-inducing and replicative capacities, the combination of both amino-terminal and carboxy-terminal mutations converted the 168.1 non-syncytium-inducing, low-replicating phenotype to an MN-like syncytium-inducing, high-replicating phenotype. This again demonstrates that the V3 region affects the syncytium-inducing and replication capacities of HIV-1 and that besides the tip of the loop, both sides of the flanking V3 sequences are involved. The syncytium-inducing phenotype as observed in SupT1 cells correlates well with the level of replication of a chimeric HIV-1. Conversely, when assayed in A3.01 cells which do not support syncytium formation, the chimeric viruses showed a replication comparable to that in SupT1 cells. We propose that both the syncytium-inducing phenotype and the high-replicating phenotype are the result of a V3-dependent process involving the uptake of HIV-1 by its host cell.

Our data show that the content of the syncytium-inducing and high-replicating phenotype correlates with the content of basic amino acids within the V3 loop. However, the correlation is not absolute, and the structure of the V3 loop will most likely also play a role, as demonstrated, for instance, by the chimeric viruses 168.1 with GQRR and 168.1 with GPRR. These both have the same charge, but the chimeric virus with GQRR is superior to the chimeric virus with GPRR in replication in SupT1 cells. Notwithstanding the importance of the structure, a strongly positive charge of the V3 loop seems to promote both the syncytium-inducing capacity and the level of replication of HIV-1. Therefore, we and others (2) speculate that the V3 loop interacts with a negatively charged protein or protein domain. In support of this hypothesis the negatively charged dextran sulfate, which is a potent inhibitor of HIV-1 replication, inhibits binding of antibodies to the V3 region and is thereby probably mimicking the natural substrate of the V3 loop (2). The protein with which V3 interacts could be either a putative second receptor or the negatively charged CDR3 region of the CD4 molecule. In line with the latter hypothesis, monoclonal antibody L71, which binds to the CDR3 region of CD4, can block viral infection and syncytium formation but not the binding of CD4 to gp120 *per se* (28). Moreover, a single point mutation (Gly to Glu) in the CDR3 region of chimpanzee CD4 can change HeLa-chimpanzee CD4 cells from non-syncytium-supporting cells to cells supporting syncytium formation upon HIV-1 infection (3). In this hypothesis, binding of gp120 to CD4 first takes place via interaction of the classic CD4 binding domain of gp120 (19) to the CDR2 region of CD4. This primary interaction induces a conformational change in gp120 (and maybe CD4 as well). The conformational change in gp120 and maybe in CD4 should then allow the interaction between the positively charged gp120 V3 region and the negatively charged CDR3 region of CD4 (29). Subsequently, fusion and entry of HIV-1 could occur. At present, we cannot discriminate between the second receptor and the CD4 interaction hypotheses.

The results of this study demonstrate that the V3 region of gp120 is an important determinant of syncytium-inducing capacity and replication of HIV-1. We speculate that an interaction between the V3 region of gp120 and a negatively charged protein or protein domain mediates the entry of HIV-1 in its host cell.

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